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14. ABSTRACT

During the second year of this project, we have made significant progress in several of our proposed tasks. We found that both TUSC4 and PRMT5 may function as RSR genes through their activities in facilitating HR DNA repair. Both TUSC4 and PRMT5 inhibit cell proliferation and cellular transformation, and may function as potential tumor suppressor genes in breast cancer. DNA2 is also involved in HR repair in response to RSR. However, unlike TUSC4 or PRMT5, DNA2 may exert an oncogenic function in cancer cells.

In addition, we identified and validated APP as an RSR-defect-specific membrane protein and have successfully conjugated APP antibody to hollow gold nanoparticles, an important step for developing nano-imaging and nano-targeting in the future. We also identified additional RSR-defect-specific membrane molecules by SILAC. We will seek to determine if any of these molecules may serve as a better marker than APP for detecting and targeting RSR-defect cells.

Finally, using Prestwick chemical library, we have successfully completed our screen for drugs that may specifically target on RSR-defect cells. We will evaluate the sensitivity and specificity of these drugs on killing the RSR-defect cells soon.

15. SUBJECT TERMS
Replication stress response, gene signature, SILAC, nanoparticles, drug screening

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Table of Contents

	Page
Introduction…….	**4**
Body……..	**4-11**
Key Research Accomplishments……..	**11**
Reportable Outcomes……	**11**
Conclusion……	**11**
References……	**11-12**
Appendices……	**(1 paper)**

INTRODUCTION

In both precancerous breast lesions and breast cancer, hyperproliferative activity due to oncogene activation or loss of tumor suppressor genes induces stalling and collapse of DNA replication forks, which in turn activates the replication stress response (RSR) to maintain genome integrity [1-4]. RSR is a subset of the DNA damage response that safeguards the replication process [5]; defects in RSR allow the survival and proliferation of genomically unstable cells, ultimately leading to breast cancer [4-6]. Since the initial RSR defects occur before cancer develops, RSR defects can serve as a powerful biomarker to predict the risk of cell transformation. Importantly, the presence of RSR defects distinguishes premalignant lesions and breast cancer from normal tissues, which makes these defects effective targets for both breast cancer prevention and breast cancer treatment. This project is to use cutting-edge technologies to characterize novel RSR genes and their functions in tumor suppression; identify gene signature and membrane proteins associated with defective RSR; identify drugs that target these defects; and develop RSR-defect-targeting nanoparticles for diagnostic imaging, prevention, and treatment of breast cancer.

As demonstrated in our first-year progress report, we have established a highly valuable breast cell model for the study of oncogene-induced replication stress. In this cell model, we induce cyclin E expression and place replication stress by adding doxycycline to the medium. Using this system, we sought to validate the five RSR candidate genes from our previous siRNA screen, and we found TUSC4 and PRMT5 to exhibit the characteristics of known RSR genes. Since intact RSR is critical for suppressing cellular transformation, we further investigated how these two genes mechanistically participate in RSR and if they function as novel tumor suppressor genes in breast cancer.

DNA2 was not initially validated as an RSR gene using our cyclin E-inducible model. However, we also found a role of DNA2 in the process of replication stress response. Notably, unlike TUSC4 and PRMT5, which function as potential tumor suppressor genes, we found DNA2 to be overexpressed in cancer cells and is required for supporting cancer cells to overcome the replication stress [7 and Appendix].

During first year of this project, we successfully established a gene-expression signature that may represent cells lacking RSR. Based on this RSR gene signature and SILAC (Stable isotope labeling by amino acids in cell culture) proteomics done in the second year, we sought to identify and validate the potential RSR-defect membrane markers for future nano-imaging and nano-targeting development.

The progress of our second year research is described below.

BODY

The tasks involved in our second-year research include: Task 1b,c, Task 2b,c,d, Task 3a and Task 4a from our final version of Statement of Work.

<u>Task 1b. To study how these five RSR candidates mechanistically participate in the RSR network.</u>
As demonstrated in our first-year progress report, among the five potential candidates, we have validated the function of TUSC4 and PRMT5 in response to replication stress. During the second year, we sought to mechanistically investigate how these two genes function in RSR. The replication stress caused by oncogene activation is commonly associated with stalling of replication forks and consequently leads to replication-associated double-strand breaks (DSBs). In response to these DNA breaks, many RSR genes are activated to facilitate the repair of DSBs by homologous recombination

(HR). To determine if TUSC4 and/or PRMT5 are required for HR repair, we generated a stable HR reporter cell line in MCF10A cells. We analyzed the HR repair capacity of these cells with or without knockdown of TUSC4 or PRMT5 using HR repair analysis [8]. In brief, the DR-GFP plasmid (Figure 1, left) was integrated into genomic DNA to generate the stable DR-GFP-10A cell line. SceGFP contains an I-SceI endonuclease site, which abolishes GFP expression. iGFP is a truncated GFP, which contains homologous sequence for SceGFP. Expression of I-SceI induces a single double-strand break (DSB) in the genome. When this DSB is repaired by HR, GFP expression is stored and can be analyzed by flow cytometry to determine HR repair efficiency. Using this delicate assay, we found that knockdown of TUSC4 or PRMT5 had a significant decrease in HR repair-induced GFP-positive cells, indicating defective HR repair in breast cells due to loss of these two genes (Figure 1, right). These results suggest that TUSC4 and PRMT5 may function as RSR genes, at least in part, through their roles in facilitating HR DNA repair.

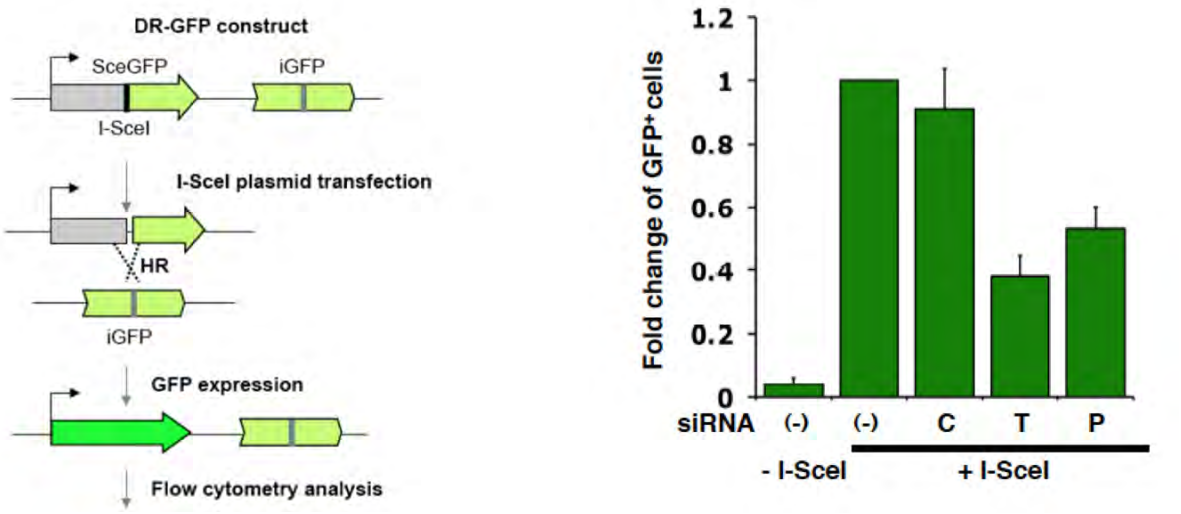

Figure 1. TUSC4 and PRMT5 are required for HR DNA repair in MCF10A cells. (Left) Schematic diagram of HR assay. The DR-GFP reporter substrate was integrated into cellular genomic DNA. SceGFP contains an I-SceI endonuclease site within the coding region, which abolishes GFP expression. iGFP is a truncated GFP that contains homologous sequence for SceGFP. Expression of I-SceI induces a single DSB in the genome. When this DSB is repaired by HR, the expression of GFP is restored and can be analyzed by flow cytometry to determine the efficiency of HR repair. (Right) Defective HR repair in TUSC4- or PRMT5-depleted MCF10A cells. MCF10 cells were transfected with nontargeting siRNA (C), TUSC4 siRNA (T), or PRMT5 siRNA (P) upon induction of DSBs by I-SceI. The graph shows a quantitative summary of at least three independent experiments. Each value is relative to the percentage of GFP-positive (GFP+) cells in I-SceI-transfected cells without siRNA transfection, which was set to 1.

Interestingly, although not being validated as an RSR gene by our cyclin E-induced cell model, we also found DNA2 to be implicated in oncogene-induced replication stress response. As reported in our recent Cancer Research paper [7 and appendix], we found that DNA2 can facilitate HR to repair replication-associated DNA DSBs and provide cancer cells with survival advantages under conditions of replication stress. We also found the nuclease activity of DNA2 being required for DSB end resection, which allowed subsequent recruitment of RPA and RAD51 to repair DSBs and restart replication. Importantly, DNA2 expression was significantly increased in breast cancer as well as other types of human cancer [7 and appendix], and exhibited a unique oncogenic property that was distinct from the tumor suppressive functions of TUSC4 and PRMT5.

We also sought to determine how TUSC4 and PRMT5 participated in homologous recombination DNA repair. We tested whether TUSC4 or PRMT5 interacted with the known key regulators in HR pathway. We generated the stable cell lines in MCF10A that expressed Flag-TUSC4, Flag-PRMT5 or the control 3xFlag tag. As shown in the Figure 2, by immunoprecipitation, we found that TUSC4 physically associates with both ATM and ATR, two major kinases that control DNA damage responses, including HR repair. Therefore, it is possible that TUSC4 may interact with ATM/ATR to facilitate HR DNA repair in response to replication stress.

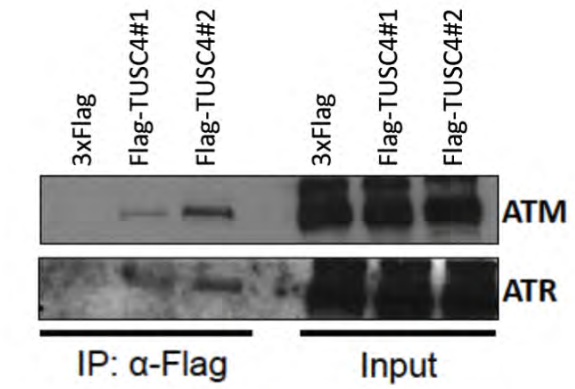

Figure 2. TUSC4 associates with ATM and ATR. Lysates from the control or two TUSC4 stable cell lines were immunoprecipitated by anti-Flag antibody and probed with anti-ATM or anti-ATR antibody by Western blot analysis.

We didn't detect any direct binding between PRMT5 and ATM or ATR. Instead, we found interaction between PRMT5 with RPA, a single-strand DNA binding protein known to be required for ATR-mediated DNA damage response (Figure 3). Therefore, we suspected that PRMT5 may participate in ATR pathway in response to replication stress through RPA. We will continue to investigate if and how the bindings of TUSC4 and PRMT4 with these DNA damage response proteins affect their functions in HR and RSR.

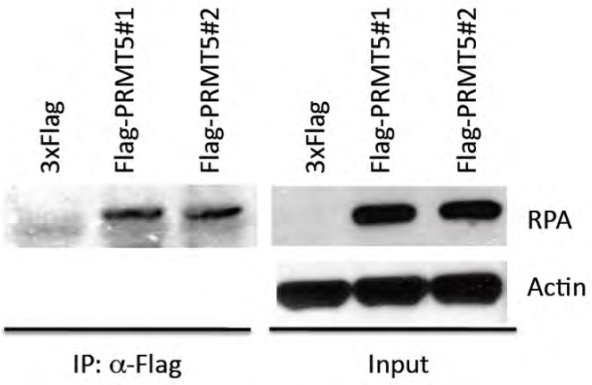

Figure 3. PRMT5 associates with RPA. Lysates from the control or two PRMT5 stable cell lines were immunoprecipitated by anti-Flag antibody and probed with RPA antibody by Western blot analysis. Equal amount of Actin detected in all inputs indicated the equal usage of lysates during the immunoprecipitation process.

<u>Task 1c. To determine whether any of these five RSR candidates functions as tumor suppressor gene in breast cancer.</u>

To assess if TUSC4 or PRMT5 functions as tumor suppressor genes in breast cancer, we sought to determine whether depletion of these two genes individually would lead to cellular transformation in vitro and tumorigenicity in mice. To this end, we first knocked down either TUSC4 or PRMT5 expression in MCF10A cells by shRNA constructs. We then examined if knockdown of these two genes enhanced the ability of cell growth on plates and in soft-agar. As shown in Figure 4, depletion of TUSC4 or PRMT5 expression increased the cell growth using clonogenic assay, indicating the anti-proliferative activity of these two genes in breast cells.

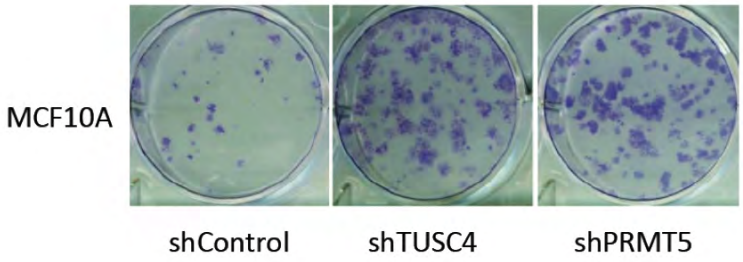

Figure 4. Knockdown of TUSC4 or PRMT5 enhances normal breast cell growth. Representative images of clonogenic assay that measures cell growth in MCF10A cells transfected with the indicated shRNAs.

We also grew these cells in soft-agar as a standard in vitro transformation assay. As shown in Figure 5, compared to the cells transfected with the control shRNA, the MCF10A cells with either TUSC4 or PRMT5 knockdown significantly gained their ability to form colony in soft agar, indicating their increased transformation phenotype.

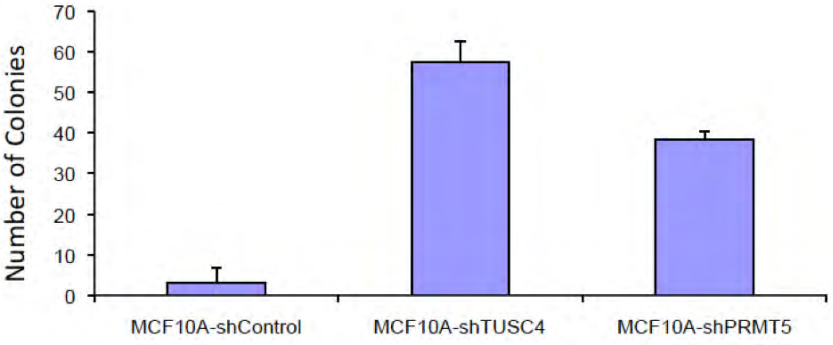

Figure 5. TUSC4 and PRMT5 both suppress transformation of MCF10A cells. Knockdown of TUSC4 or PRMT5 induced anchorage-independent growth of MCF10A cells.

In addition to the in vitro proliferation and transformation assays, we will inject these cell lines into mammary fat pad of nu/nu mice to determine if knockdown of TUSC4 or PRMT5 in MCF10A cells will enhance their growth as mammary tumors in mice.

Due to the removal of Dr. Meric-Bernstam as a collaborator during the contract negotiation, we therefore didn't further pursue our study on analyzing the potential clinical reference of our RSR defect gene signature at this stage of project.

<u>Task 2b. To investigate whether the membrane proteins highly expressed in the RSR-defect signature are specific to RSR-defective cells.</u>

Our RSR-defect gene expression signature includes three genes that encode membrane proteins. Among these genes, we are mainly focused on a gene called Amyloid protein precursor (APP) because this gene was also independently identified by SILAC as one of top-ten membrane proteins in RSR-defect cells (see Table 1 below).

As shown in Figure 6A, we detected the membrane localization of APP by immunofluorescent staining. More importantly, knocking down various known RSR genes, such as ATM, ATR, CHEK1, CHEK2, led to the increase of APP expression (Figure 6B). These important results clearly validated APP as a specific RSR-defect membrane marker.

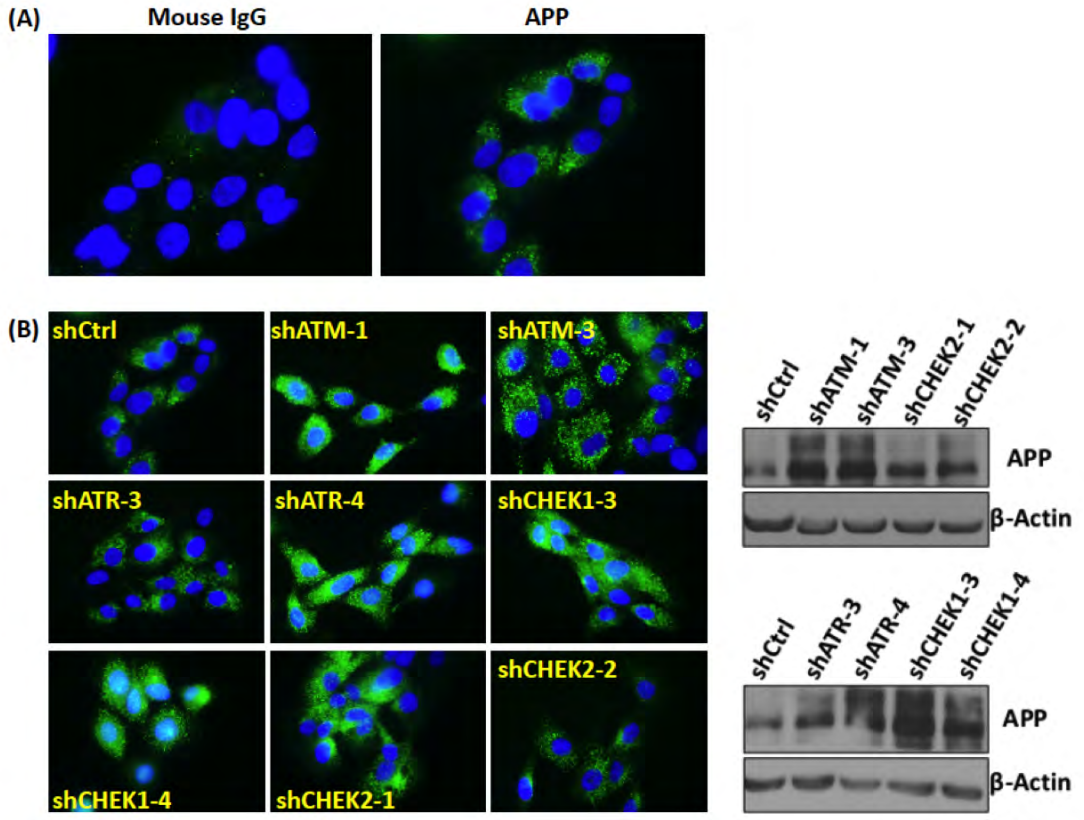

Figure 6. Amyloid protein precursor (APP) is a membrane protein that is upregulated when RSR genes are depleted. (A) MCF10A cells were stained with mouse IgG or anti-APP antibody in the absence of surfactants. Nucleus was stained by DAPI. (B) Left panel: MCF10A cells with stable knockdown of various RSR genes including ATM, ATR, CHEK1 and CHEK2 were stained by anti-APP antibody in the absence of surfactants. Right panel: The protein expression level of APP in MCF10A cells with stable knockdown of variable RSR genes

Since intact RSR is a critical barrier to prevent cancer development, and APP expression increases in RSR-defect cells, we expected to detect higher APP expression in breast cancer compared to normal breast tissue. By analyzing breast tissue arrays from US Biomax Inc, we found APP expression is upregulated in both hyperplastic breast tissue and breast cancer compared to normal breast tissue (Figure 7).

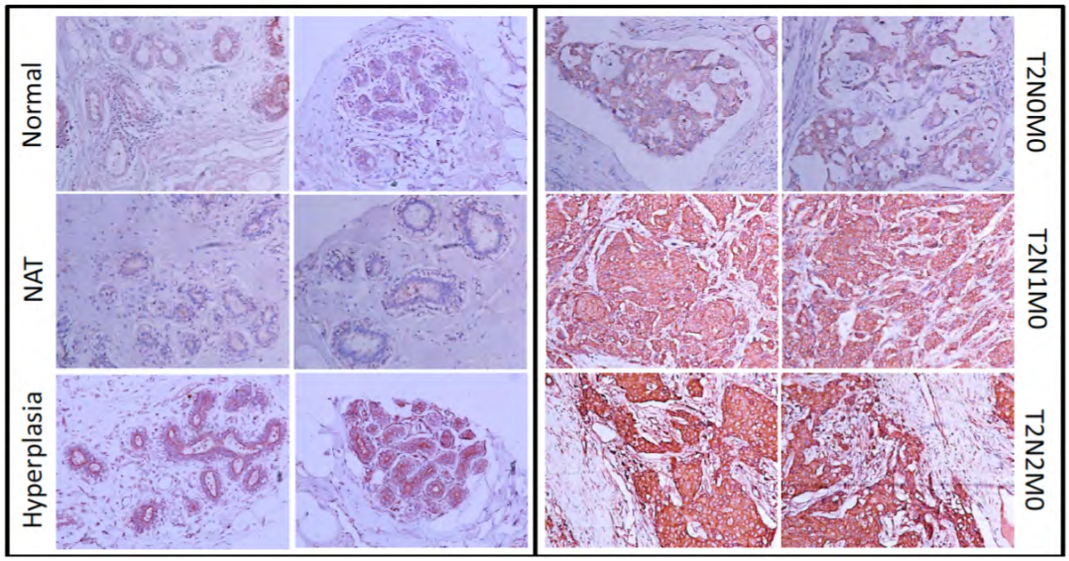

Figure 7. Amyloid protein precursor (APP) is upregulated in hyperplasic breast tissue and breast cancer. Normal breast tissue, cancer adjacent normal breast tissue (NAT), hyperplasic breast tissue, and different stages of invasive ductal carcinomas were performed with immunohistochemistry by staining with anti-APP antibody. Different stages of breast cancer tissues were presented by TNM grading. T2 indicates that tumor has invaded into the muscularis propria. N0 indicates regional lymph node metastasis. N1 indicates metastasis in 1 to 3 regional lymph nodes. N2 indicates metastasis in 4 or more regional lymph nodes. M0 indicates no distant metastasis.

Task 2c. To identify RSR-defect-specific membrane proteins by SILAC.

In addition to analyzing the expression of membrane proteins identified in our RSR-defect gene signature, we performed SILAC proteomics analysis to identify additional RSR-defect-specific membrane proteins. SILAC is a mass spectrometry-based technique that is designed to identify the differential expression of proteins in two different cell populations [9,10]. We isolated membrane proteins from either cyclin E-induced MCF10A control cells or the same cells with ATR knockdown by shRNA. By comparing the differential expression between these two populations by mass spectrometry, we identified many proteins highly expressed in the ATR knockdown cells (i.e. RSR-defect) than the control. The Table 1 lists the top-ten candidates.

TMC4
ERMAP
APP
FBXL20
GRINA
ITGB2
LPPR2
MKNK2
MME
PLXNB2

Table 1. Top-ten RSR-defect-specific membrane protein candidates identified by SILAC analysis.

Task 2d. To validate the RSR-defect-specific membrane proteins.

As shown in the Task 2a above, we have successfully validated APP as an important RSR-defect-specific membrane protein. We will continue to validate the rest of the candidates using the specific antibodies against these molecules in our RSR model system.

Task 3a. To screen for drugs and compounds that specifically target on cells with RSR-defect signature.

We sought to identify drugs that target RSR-defective breast cancer cells. Two different chemical libraries—Prestwick and Chembridge—have been chosen for the screen. The Prestwick chemical library contains 1120 small compounds selected for their high chemical and pharmacological diversity and their known bioavailability and safety in humans. The Chembridge library includes 30,000 compounds. These screens were originally planned to be performed through the High-Throughput Screening Core Facility at the John S. Dunn Gulf Coast Consortia for Chemical Genomics in Houston. However, due to a long list of projects waiting for their service, to avoid a long delay of our progress, we decided to screen Prestwick chemical libraries manually in our lab because of its relatively limited drug number. We screened for the drugs that could preferentially kill RSR-defect-cyclin E overexpressing MCF10A cells (i.e., knockdown of ATM or ATR) than the RSR-intact cells (treated with non-targeting shRNA). The top-10 drugs identified from our screen are listed in the Table 2.

Table 2. Drug candidates that preferentially kill RSR-defect cells.

Benzethonium chloride
Sanguinarine
Doxorubicin hydrochloride
Selegiline hydrochloride
Mitoxantrone
Alexidine dihydrochloride
Etoposide
Paclitaxel
Digoxin
Mitoxantrone dihyrochloride

We have tried to screen the Chembridge library as well. However, due to the high number of compounds in the library, we encountered technical difficulty in completing our screen manually. We will, therefore, wait for the availability in the High-Throughput Screening Core Facility at the John S. Dunn Gulf Coast Consortia for our compound screening.

Task 4a. To develop nano-imaging technology to detect RSR-defective breast cancer cells through binding of nano-imaging particles to the RSR-defect-specific membrane proteins.

In collaboration with our colleague Dr. Chun Li, an outstanding leader in nanotechnology, we aimed to develop nanoparticles that can carry in vivo imaging agents to target breast cancer cells with RSR-defect-specific membrane proteins for cancer detection. During the first year of this project, Dr. Li has successfully conjugated anti-EGFR antibody to nanoparticles to demonstrate the feasibility of this technique. During the second year of this project, we have been working on the conjugation of antibody against one of our identified RSR defective markers, APP. Hollow gold nanoparticles (HAuNP) were synthesized by Dr. Li's lab. APP antibody (clone 22C11, Millipore Corporation, Billerica, MA) was first incubated with N-Succinimidyl-S-Acetyl-Thioacetate (SATA) to convert primary amine groups of APP antibody into protected thiol (-SH) group. This process prevents self-aggregation of APP antibody and provides thiol group for APP/HAuNP conjugation. After SATA/APP antibody reaction, excess SATA was removed by using desalting column (PD MiniTrap G10, GE Healthcare, Piscataway, NJ). SATA-APP-antibody solution was then mixed with hydroxylamine and HAuNP. Hydroxylamine activates both thiol groups on SATA-APP-antibody and HAuNP. This reaction allows SATA-APP-antibody to conjugate to HAuNP by forming disulfide (-S-S-) bonds. The final product of HAuNP-APP-antibody was incubated in PBS (pH 7.4) solution. We confirmed the success of conjugation by SDS PAGE analysis (Figure 8).

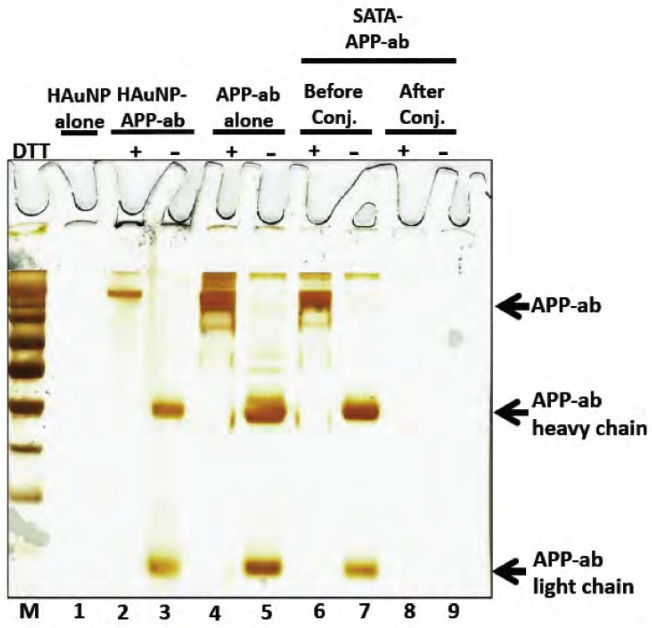

Figure 8. The examination of efficiency of APP antibody (APP-ab) conjugated to hollow gold nanoparticles (HAuNP). APP-ab was chemically modified by N-succinimidyl S-acetylthioacetate (SATA) before conjugated to HAuNP. APP-conjugated HAuNP (HAuNPAPP-ab) was applied to lane 2 and 3 on SDS PAGE in the presence and absence of redox reagent Dithiothreitol (DTT), respectively. APP–ab (1μg) was applied to lane 4 and 5. Land 6 to 9 indicate the binding efficiency of SATA-APP-ab/HAuNP conjugation.

KEY RESEARCH ACCOMPLISHMENTS

(1) We identified the roles of two novel RSR genes, TUSC4 and PRMT5, in homologous recombination (HR) DNA repair. The HR function of TUSC4 may be mediated through its interaction with ATM/ATR. PRMT5, instead of direct binding to ATM/ATR, may participate in ATR pathway through its binding to RPA.
(2) We demonstrated the functions of TUSC4 and PRMT5 in suppressing breast cell proliferation and cellular transformation.
(3) We identified a role of DNA2 in homologous recombination DNA repair in response to replication stress. Unlike TUSC4 and PRMT5, DNA2 appears to exert an oncogenic function that helps breast cancer cells to tolerate high replication stress.
(4) We identified and validated APP as an RSR-defect-specific membrane protein.
(5) By SILAC analysis, we identified many potential RSR-defect-specific membrane protein candidates.
(5) Using Prestwick chemical library, we successfully completed our screen for drugs that specifically target on RSR-defect cells
(6) We successfully conjugated APP antibody to hollow gold nanoparticles (HAuNP).

REPORTABLE OUTCOMES

Part of the our study has led to a publication in Cancer Research (7 and appendix) and invited presentations at both Baylor College of Medicine in Houston and National Health Research Institutes in Taiwan.

CONCLUSION

During the second year of this project, we have made significant progress in several of our proposed tasks. We found that both TUSC4 and PRMT5 may function as RSR genes through their activities in facilitating HR DNA repair. Both TUSC4 and PRMT5 inhibit cell proliferation and cellular transformation, and may function as potential tumor suppressor genes in breast cancer. DNA2 is also involved in HR repair in response to RSR. However, unlike TUSC4 or PRMT5, DNA2 may exert an oncogenic function in cancer cells.

In addition, we identified and validated APP as an RSR-defect-specific membrane protein and have successfully conjugated APP antibody to hollow gold nanoparticles, an important step for developing nano-imaging and nano-targeting in the future. We also identified additional RSR-defect-specific membrane molecules by SILAC. We will seek to determine if any of these molecules may serve as a better marker than APP for detecting and targeting RSR-defect cells.

Finally, using Prestwick chemical library, we have successfully completed our screen for drugs that may specifically target on RSR-defect cells. We will evaluate the sensitivity and specificity of these drugs on killing the RSR-defect cells soon.

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Appendix

Molecular and Cellular Pathobiology

Cancer Research

Human Nuclease/Helicase DNA2 Alleviates Replication Stress by Promoting DNA End Resection

Guang Peng[1], Hui Dai[2], Wei Zhang[1], Hui-Ju Hsieh[1], Mei-Ren Pan[2], Yun-Yong Park[2], Robert Yu-Lin Tsai[4], Isabelle Bedrosian[3], Ju-Seog Lee[2], Grzegorz Ira[5], and Shiaw-Yih Lin[2]

Abstract

In precancerous and cancerous lesions, excessive growth signals resulting from activation of oncogenes or loss of tumor suppressor genes lead to intensive replication stress, which is recognized by a high level of replication-associated DNA double-strand breaks (DSB). However, the molecular mechanism by which cells alleviate excessive replication stress remains unclear. In this study, we report that the human nuclease/helicase DNA2 facilitates homologous recombination to repair replication-associated DNA DSBs, thereby providing cells with survival advantages under conditions of replication stress. The nuclease activity of DNA2 was required for DSB end resection, which allowed subsequent recruitment of RPA and RAD51 to repair DSBs and restart replication. More importantly, DNA2 expression was significantly increased in human cancers and its expression correlated with patient outcome. Our findings therefore indicate that enhanced activity of DSB resection likely constitutes one mechanism whereby precancerous and cancerous cells might alleviate replication stress. *Cancer Res; 72(11); 2802–13. ©2012 AACR.*

Introduction

Tumorigenesis is a multistep process by which normal cells successively acquire genetic alterations (1). Analyses of human tumors have shown that the presence of DNA damage, particularly DNA double-strand breaks (DSB), distinguishes precancerous lesions and cancer from normal tissues (2, 3). Recent studies have indicated that oncogene-induced replication stress underlies DSB formation (4, 5). Specifically, activation of oncogenes provides cells with sustained proliferative signaling and leads to inappropriate DNA replication, which results in fork collapse and DSBs (6, 7).

There are 2 pathways for repairing DSBs: homologous recombination (HR) and nonhomologous end-joining (NHEJ; ref. 8). NHEJ involves direct ligation of broken ends and primarily occurs in the G_1 phase of the cell cycle. HR is considered to be more error-free repair mechanism that copies sequences from the homologous template to repair damaged DNA. It predominantly occurs in the S- and G_2 phases, when preferable template, sister chromatids, are available. Thereby, HR is a key pathway for repairing stalled and collapsed replication forks that occur spontaneously or are induced by topoisomerase I inhibitors such as camptothecin or polymerases inhibitors such as aphidicolin (9). Repair of chromosomal breaks by HR is initiated by resection of the 5′ strands that generates 3′ ssDNA tails at DSB ends (10, 11). Resection allows loading of single-strand–binding protein RPA that is further replaced by key enzyme in HR, strand exchange protein RAD51 with the help of BRCA2 (12–14). RAD51 mediates homology search and strand invasion at template sister chromatid, which is followed by DNA synthesis and resolution of recombination intermediate that restores replication fork. Depletion of RAD51 leads to accumulation of unrepaired DSBs and cells death showing the importance of HR for repair of spontaneous DNA breaks occurring during replication (15).

Continuous formation of DSBs induced by replication stress activates DNA damage response (DDR), which induces senescence or apoptosis and thus prevents precancerous lesions from progressing to malignant lesions (6, 7). Impairment of DDR (e.g., through loss of expression of signaling kinases ATM or CHK2) can lead to a breach of this anti-cancer barrier and tumor progression (4, 5). However, it remains unclear that how precancerous and cancerous cells could cope with increased replication-associated DSBs and maintain their hyperactive DNA replication status.

In this study, we used a proteomic approach to investigate protein components preferentially associated with replication forks in the presence of oncogene activation. Here, we report that human nuclease/helicase DNA2 is overexpressed in a variety of cancers and it plays an important role in alleviating

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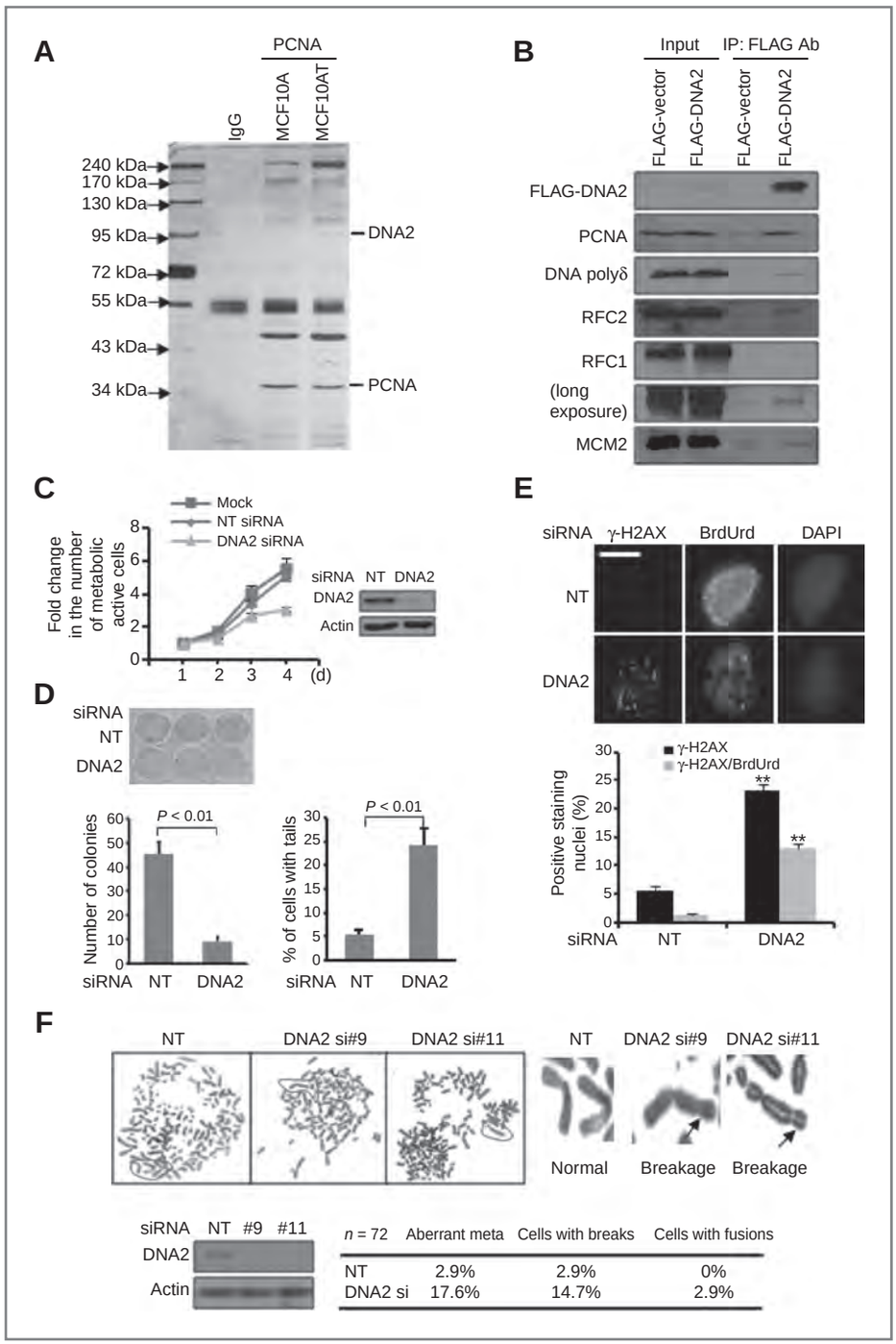

Figure 1. DNA2 associates with replication forks and regulates replication-associated DSBs. A, cell extracts were prepared from MCF10A and MCF10AT cells for immunoprecipitation (IP). B, co-IP of DNA2 in U2OS cells transfected with Flag-vector or Flag-DNA2. C and D, U2OS cells were transfected with control siRNA (NT) or DNA2 siRNA (SMARTpool). Each value represents the mean ± SEM from 3 independent experiments. C, MTT assay each day relative to day 1. D, left, clonogenic assay. Right, quantitative analysis of comet assay. At least 100 cells were scored in each sample. U2OS cells were transfected with DNA2 siRNA. E, seventy-two hours later, cells were pulsed with BrdUrd (10 μmol/L) for 30 minutes and then stained. Top, representative images (scale bar, 5 μm). Bottom, at least 50 cells were scored in each sample (**, $P < 0.01$). F, top, cells were analyzed by metaphase spreads. Bottom, quantitative summary. Western blot analyses showing effective DNA2 knockdown are shown next to the graphs. Ab, antibody; DAPI, 4′,6-diamidino-2-phenylindole.

replication stress likely by promoting DNA end resection and HR repair of replication-associated DSBs.

Materials and Methods

Cell culture

U2OS and MCF10A cells were purchased from American Type Culture Collection (ATCC). U2OS cells were maintained in McCoy 5A medium supplemented with 10% FBS. MCF10A cells were cultured in mammary epithelial growth medium containing insulin, hydrocortisone, EGF, and bovine pituitary extract purchased from Clonetics. MCF7 cells were maintained in Dulbecco's Modified Eagle's Media (DMEM; Cellgro) with 10% FBS. Cell lines were validated by short tandem repeat (STR) DNA fingerprinting using the AmpFISTR Identifiler Kit according to manufacturers' instructions (Applied Biosystems

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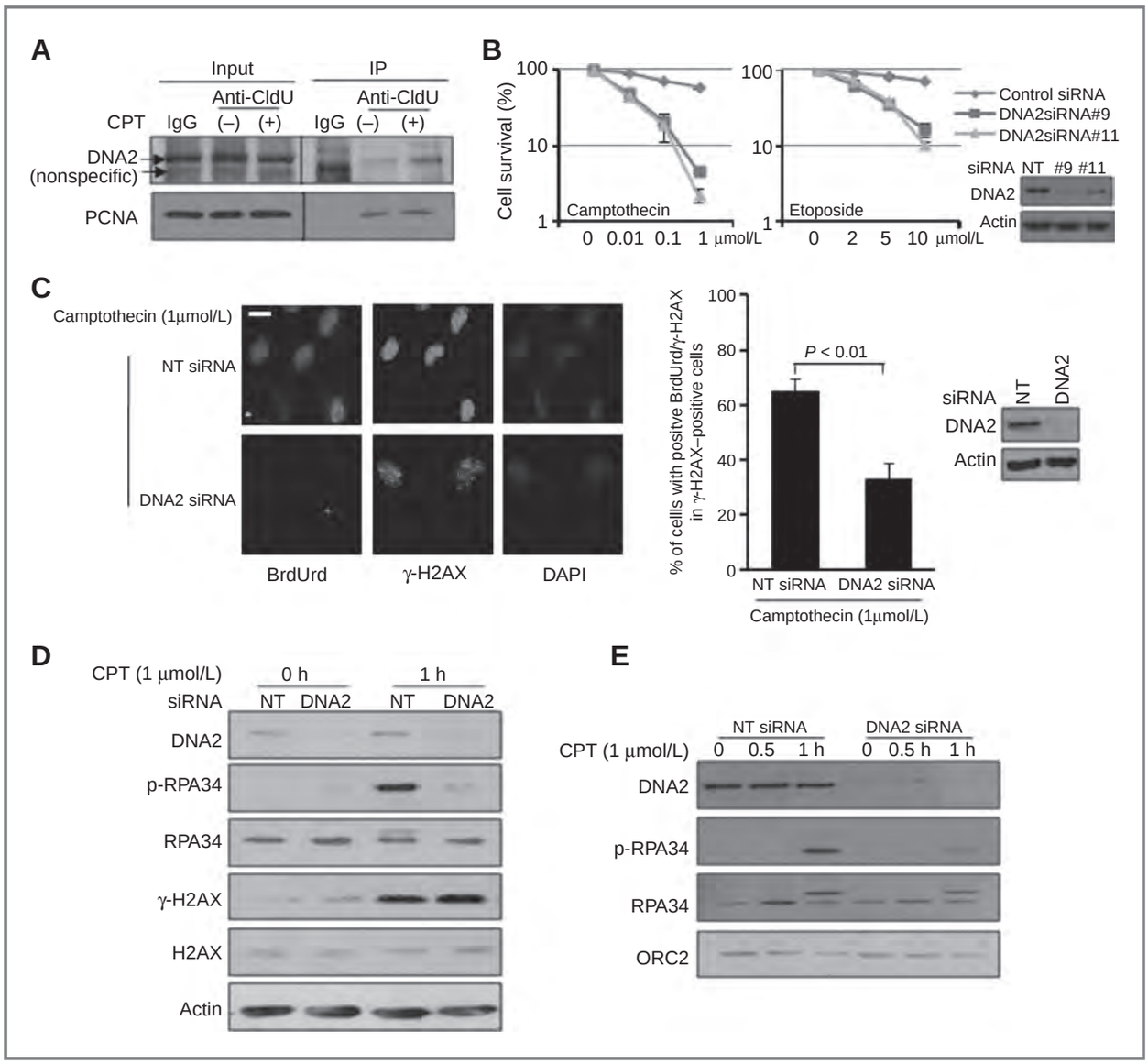

Figure 2. DNA2 accumulates at restarted replication forks and promotes DSB end resection. A, U2OS cells were treated with camptothecin (CPT; 1 μmol/L) for 1 hour and pulsed with CldU for 30 minutes before immunoprecipitation (IP) analysis. PCNA was used as a control. B, U2OS cells were transfected with control siRNA or DNA2 siRNAs (#9 and #11) and then treated with CPT (left) or etoposide (right). U2OS cells were transfected with control siRNA or DNA2 siRNAs. C, forty-eight hours later, cells were treated with or without CPT (1 μmol/L) for 1 hour. Cells were labeled with BrdUrd (10 μmol/L) for 30 minutes. γ-H2AX and BrdUrd were co-stained in native conditions. Left, representative images (scale bar, 5 μm). Right, at least 50 cells were scored in each sample. Each value represents the mean ± SEM from 2 independent experiments. D and E, cells were treated with CPT (1 μmol/L) for 1 hour. Total cell lysates (D) and chromatin-enriched fractions (E) were analyzed. Western blot analyses showing effective DNA2 knockdown are shown next to the graphs. DAPI, 4′,6-diamidino-2-phenylindole; T, total.

catalogue no. 4322288). The STR profiles were compared with known ATCC fingerprints, with the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (Nucleic Acids Research 37:D925-D932 PMCID: PMC2686526), and with the MD Anderson fingerprint database. The STR profiles matched known DNA fingerprints or were unique. MCF10A cells with stable cyclin E expression were generated by transfection with pcDNA3.1-cyclin E construct and maintained in the presence of 100 μg/mL G418. A series of pancreatic cell lines (HPDE, PD90, PD78, PD77, and PD74) representing RAS-mediated pancreatic cancer development were kindly provided by Dr. Michel J. Quellette (University of Nebraska Medical Center, Omaha, NE). Cells were maintained as previously described (16).

Antibodies, immunoprecipitation, chromatin fractionation, and Western blot analysis

Antibodies used in the experiments include DNA2, H2AX, and rat anti-BrdU antibodies (Abcam); γ-H2AX (Upstate Biotechnology); RPA34 (Neomarkers); p-RPA34 (Bethyl

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Laboratories); Chk1, p-Chk1 (Ser345), and p-p53 (Cell Signaling); ORC2, PCNA, DNA polymerase δ, RFC2, RFC1, MCM2, and p21 (Santa Cruz Biotechnology); and Rad51 (Ab-1), mouse anti-BrdU, and p53 antibodies (Calbiochem). The immunoprecipitation with anti-Flag affinity beads was done in U2OS cells transiently transfected with Flag-tagged plasmids. Forty-eight hours later, whole-cell extracts were prepared with radioimmunoprecipitation assay (RIPA) buffer and immunoprecipitated with anti-Flag M2 affinity gel (Sigma) overnight. Bead-bound immunocomplexes were eluted with 3x FLAG peptide (Sigma) and subjected to SDS-PAGE. For reciprocal immunoprecipitation, whole-cell extracts were prepared in RIPA buffer as indicated above and precleaned with protein A/G plus-agarose beads (Santa Cruz). Then, cellular extracts were subjected to incubation with antibodies against proliferating cell nuclear antigen (PCNA) and DNA polymerase δ (2 μg/mg of cell lysis) overnight and then incubated with protein A/G agarose

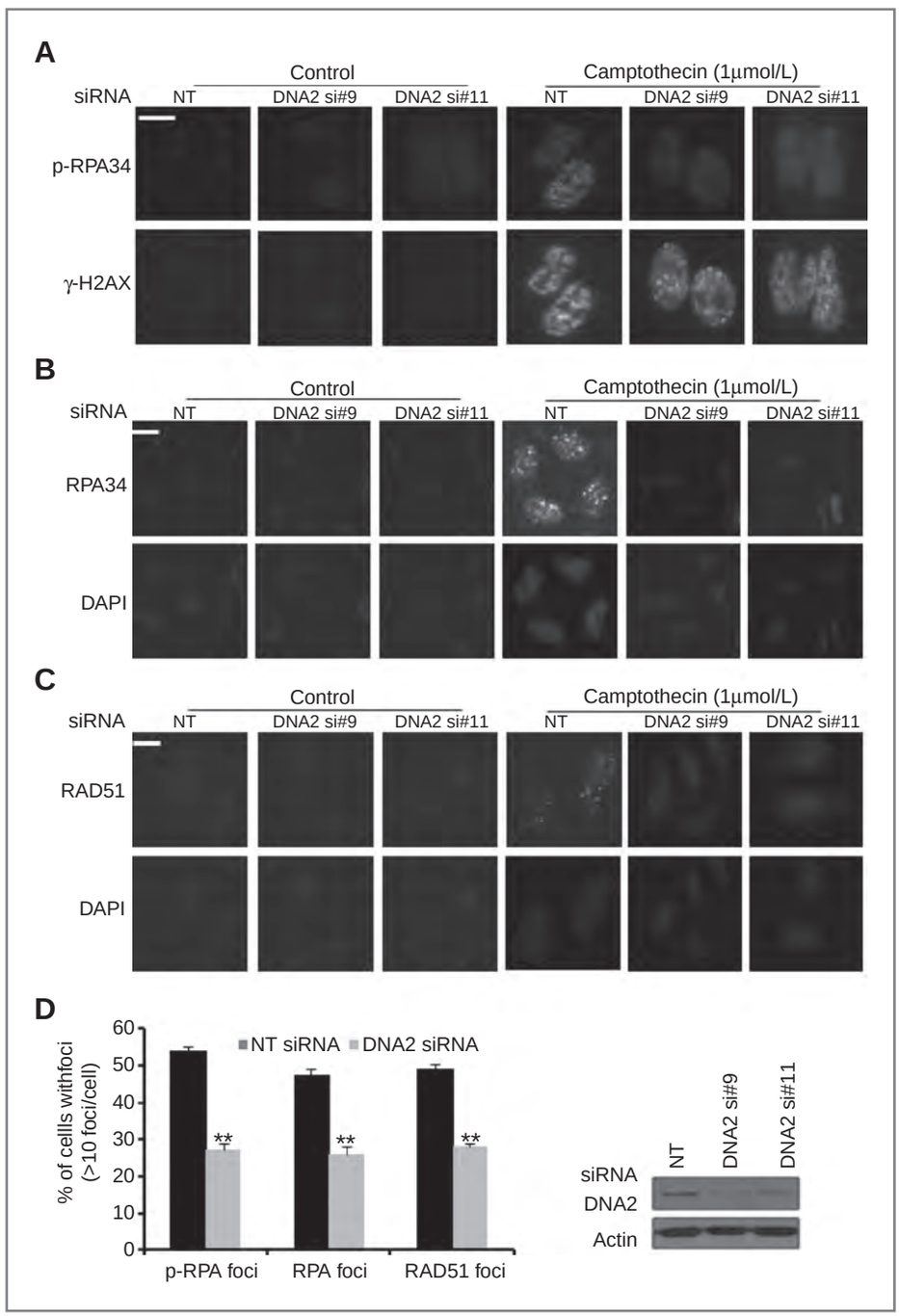

Figure 3. DNA2 depletion impairs the recruitment of RPA and RAD51 to replication-associated DSBs. U2OS cells were transfected with control siRNA or DNA2 siRNAs (#9 and #11). Forty-eight hours later, cells were treated with camptothecin (CPT; 1 μmol/L) for 1 hour and co-stained with the indicated antibodies: A, p-RPA34; B, RPA34; C, RAD51; and D, quantitative results. At least 50 cells (>10 foci per cell) in each sample were scored (**, $P < 0.01$; scale bar, 5 μm). Western blot analyses showing effective DNA2 knockdown are shown next to the graph. DAPI, 4′, 6-diamidino-2-phenylindole.

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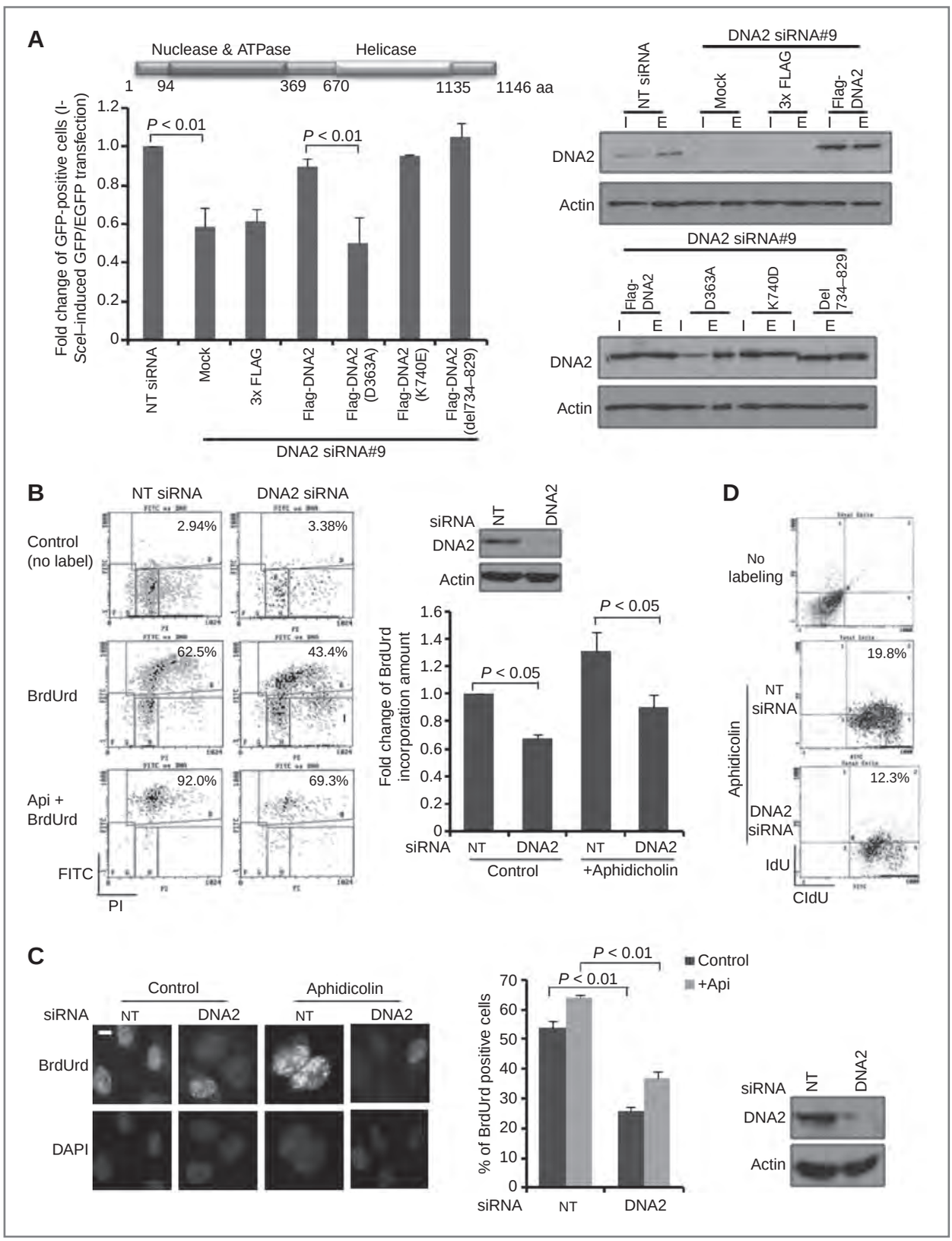

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beads for 4 hours at 4°C. The immunocomplex was eluted in loading buffer by boiling at 95°C for 5 minutes. The preparation of chromatin fractions and Western blot analysis, including the conditions for RPA analysis, were as previously described (17).

Statistical analysis

All statistical analysis was done using one-tailed Student *t* test. Additional methods are included in Supplementary Materials.

Results

DNA2 forms a complex with replication factors and prevents accumulation of replication-associated DSBs

On the basis of previous findings that the activation of oncogene *RAS* causes replication stress and replication-associated DSBs, we selected the MCF10A and MCF10AT cell lines for proteomic analysis (4, 5, 18). MCF10A cells are immortalized normal breast epithelial cells. MCF10AT cells are derived from MCF10A cells by forced expression of oncogenic *H-RAS*. MCF10A cells do not grow in immunocompromised mice. In contrast, MCF10AT cells form the lesions that resemble the progression of breast cancer. Thus, these cell lines provided us with a model system to study the genetic alterations promoting carcinogenesis initiated by oncogene activation.

Replication forks are composed of many proteins. PCNA, DNA polymerase processivity factor, encircles DNA and orchestrates replication-linked processes by recruiting crucial players to the replication fork (19). Thereby, we used PCNA as our bait to isolate replication factor–associated protein complexes. Among many known proteins involved in DNA replication including RFC factors and MCM proteins, we found that DNA2 had higher abundance in MCF10AT cells than in MCF10A cells (Fig. 1A). We then conducted immunoprecipitation analysis to confirm DNA2 as a component of replication factor–associated protein complex (Fig. 1B; Supplementary Fig. S1A). This result is consistent with previous studies in yeast and *Xenopus* showing the involvement of DNA2 helicase/nuclease in DNA replication and repair (20, 21).

In humans, DNA2 is localized in both mitochondria and nuclei (22, 23). While the mitochondrial function of DNA2 was well-documented, its nuclear functions remain unknown. We found that DNA2 depletion impaired both the number of cells with active metabolic activity and cell survival (Fig. 1C and D). By neutral comet assay, we observed that DNA2-knockdown cells had a significantly higher proportion of cells with comet tails (Fig. 1D; Supplementary Fig. S1B), suggesting that DNA2 is required to prevent accumulation of endogenous DSBs.

We next examined whether DSBs present in DNA2-deficient cells were formed in cells with ongoing replication. γ-H2AX is a marker of DSBs (24). Bromodeoxyuridine (BrdUrd) incorporation represents actively replicating cells. We found that DNA2-deficient cells had a significantly higher proportion of cells with γ-H2AX staining and 60% of these cells also showed BrdUrd incorporation (Fig. 1E), which was significantly higher than that expected from a normal replication process (25). To further confirm that DNA2-knockdown induced replication-dependent DSBs, we showed that inhibition of replication by aphidicolin significantly decreased the number of cells with positive γ-H2AX staining (Supplementary Fig. S1C). In addition, analysis of metaphase spreads showed that DNA2-deficient cells were significantly more likely to exhibit chromosomal breakage (Fig. 1F). These findings supported an important role of DNA2 in regulating accumulation of replication-associated DSBs and chromosomal stability.

DNA2 accumulates at restarted replication forks and promotes HR repair

To confirm the role of DNA2 in response to replication-associated DSBs, we pulse-labeled newly synthesized DNA with a thymidine analogue, chlorodeoxyuridine (CIdU). We conducted immunoprecipitation with antibody against CIdU to detect the protein complexes associated with newly synthesized DNA. Interestingly, we observed that the association of DNA2 with replication forks was indeed enhanced in the presence of camptothecin, which causes replication-associated DSBs (Fig. 2A). In addition, we found that DNA2-depleted cells were more sensitive to topoisomerase inhibitors generating replication-associated DSBs, camptothecin and etoposide (Fig. 2B). Together, these data suggested that DNA2 accumulates at replication forks and plays a functional role in response to replication-associated DSBs.

Several studies implicated yeast and *Xenopus* DNA2 in the initial step of HR, resection of DSBs (26–30). Also, purified human DNA2 nuclease promotes DSB end resection (31). These reports, together with our observation, led us to hypothesize that DNA2 may prevent the accumulation of replication-

Figure 4. DNA2 facilitates HR repair and replication fork restart. A, Cells containing HR repair reporter DRGFP plasmid were transfected with the indicated constructs. Left, quantitative summary of at least 3 independent experiments. Each value is relative to the percentage of GFP-positive cells in I-*Sce*I–transfected control cells, which was set to 1 and represents the mean ± SD. A schematic diagram of DNA2 structure is shown at the top. Right, Western blot analyses showing effective transfection (I, I-*Sce*I–transfected samples; E, EGFP-transfected samples as controls to normalize transfection efficiency). B, forty-eight hours after transfection with control or DNA2 siRNA, U2OS cells were untreated or treated with aphidicolin (Api; 5 μmol/L) for 17 hours. Then, cells were released and labeled with BrdUrd (10 μmol/L) for 30 minutes. Left, representative flow cytometric profile. Right, quantitative summary. Each value is relative to the percentage of BrdUrd-positive cells in cells with control siRNA transfection, which was set to 1 and represents the mean ± SD. C, U2OS cells were transfected with control siRNA or DNA2 siRNA. Forty-eight hours later, cells were treated or untreated with aphidicolin (5 μmol/L) overnight. Left, representative images of BrdUrd incorporation detected in denatured conditions 30 minutes after BrdUrd (10 μmol/L) labeling (scale bar, 5 μm). Right, at least 50 cells were scored in each sample. Each value represents the mean ± SEM from 2 independent experiments. D, U2OS cells were transfected with control or DNA2 siRNA. Forty-eight hours later, cells were labeled with CIdU (25 μmol/L) for 30 minutes and then were treated with camptothecin (1 μmol/L) for 1 hour and released in culture medium containing IdU (250 μmol/L) for 1 hour. Cells were stained with antibodies recognizing IdU and CIdU. The percentage of double-positive cells is indicated. Quantitative summary is shown in Supplementary Fig. S4A. Western blot analyses showing effective DNA2 knockdown are shown next to the graphs. DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; PI, propidium iodide.

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associated DSBs by promoting HR repair. We first examined ssDNA formation at DSBs in DNA2-depleted cells. After cells were labeled with BrdUrd, we stained nondenatured BrdUrd, which is located only at ssDNA (32, 33). We found that reduced camptothecin triggered ssDNA formation in DNA2-depleted cells (Fig. 2C), suggesting the role of DNA2 in DSB end resection. Next, we found that depletion of DNA2 impaired phosphorylation of RPA34 as measured by phospho-RPA34 antibody and by a slower migration of RPA34, but it did not affect γ-H2AX formation induced by camptothecin (Fig. 2D). In the presence of DSBs, both H2AX and RPA34 phosphorylation are regulated by kinases ATM, ATR, and DNA-PK (24, 34, 35). Because RPA is a ssDNA-binding protein and we did not observe impairment of H2AX phosphorylation, we reasoned that reduced RPA34 phosphorylation might be due to inefficient generation of ssDNA and consequently impaired recruitment of RPA34 to DSBs, where it is highly accessible to the kinases, rather than to impaired function of its upstream kinases. Indeed, chromatin fractionation assay showed that in DNA2-deficient cells, binding of both phosphorylated RPA34 and total RPA34 to chromatin was significantly reduced (Fig. 2E). We also found that DNA2 depletion resulted in reduced foci formation of both phosphorylated RPA34 and total RPA34 (Fig. 3A, B, and D), indicating impaired RPA34 recruitment to DNA damage sites. Consistent with this result, we observed significantly reduced activation of CHK1 (Supplementary Fig. S2A) and reduced foci formation of RAD51 (Fig. 3C and D), which are recruited to DSBs after RPA. These data suggested that DNA2 facilitates the recruitment of HR repair factors to DSBs.

Next, we used an I-*Sce*I–inducible recombination assay to assess whether DNA2 depletion affects HR repair (ref. 36; Supplementary Fig. S2B). We found that DNA2 knockdown significantly reduced HR repair efficiency (Fig. 4A). DNA2 contains both a nuclease/ATPase domain and a helicase domain (37). We carried out rescue experiments with RNA interference–resistant wild-type, nuclease-dead (D363A) or helicase-dead DNA2 (K740E) mutants (37). As the mitochondrial localization signal was mapped to amino acids from 734 to 829 (22), we made a DNA2 construct with a specific deletion of this region (DNA2 del734–829), which also disrupts the helicase domain. The abrogation of mitochondrial localization of this construct was confirmed by a mitochondria marker, mtHSP70 (Supplementary Fig. S2C). In the rescue experiments, we found that nuclease activity of DNA2 is required for its function in HR repair, which is independent of its mitochondria localization (Fig. 4A). The effect of DNA2 on HR repair was not due to the changes in cell-cycle distribution (Supplementary Fig. S2D). We then tested whether the DNA2 mutants would cause dominant-negative effects when they were overexpressed. As we expected, only the nuclease-dead DNA2 mutant impaired cell survival and HR repair (Supplementary Fig. S3A and S3B).

DNA2 is required for restart of replication forks

Given that DNA2 has enhanced association with replication forks and can promote repair of DSBs by HR, we proposed that DNA2 might enhance cellular tolerance of replication-associated DSBs and promote the restart of stalled or collapsed replication forks. To test this hypothesis, we carried out 3 sets of experiments. First, we analyzed the BrdUrd incorporation by flow cytometry after the release from aphidicolin treatment. We found that DNA2 deficiency impaired normal replication and had a significant impact on the restart of DNA replication after aphidicolin treatment (Fig. 4B). Second, we used immunofluorescent staining to detect BrdUrd incorporation. We observed that DNA2 was required for promoting DNA replication and restart of DNA replication after the removal of replication stress–induced factor aphidicolin (Fig. 4C). Third, we tested the efficiency of restart of replication forks in the absence of DNA2. We first labeled cells with CIdU and then labeled cells with IdU after treatment with aphidicolin. CIdU incorporation indicates unperturbed DNA synthesis (38). In contrast, IdU incorporation correlates with DNA synthesis after the release from DNA replication inhibition. Compared with control cells, DNA2-depleted cells had a reduced proportion of cells with double staining, suggesting impaired restart of replication forks after treatment with replication stress–inducing stimuli (Fig. 4D; Supplementary Fig. S4A).

DNA2 enhances cellular tolerance of replication-associated DSBs in the context of oncogene activation

Excessive growth signaling induced by oncogene activation is one of the major sources of replication-associated DSBs in cancer cells (2, 4, 5). To elucidate the pathophysiologic relevance of the function of DNA2, we asked whether DNA2 could increase cellular tolerance of replication-associated DSBs induced by oncogene activation. We ectopically expressed *H-RAS* (V12) and *DNA2* in cells and observed that H-RAS expression induced γ-H2AX and p21 formation, which was reduced in the presence of DNA2 (Fig. 5A). Next, we found that H-RAS activation led to a more than 40% reduction in the number of BrdUrd-positive cells (Fig. 5B). This impaired DNA replication was rescued by coexpression of DNA2 (Fig. 5B). We then examined BrdUrd incorporation and γ-H2AX formation to test whether DNA2 might reduce accumulation of replication-associated DSBs induced by oncogene activation. The impaired DNA replication in H-RAS–expressing cells was accompanied by increased γ-H2AX foci formation in BrdUrd-positive cells, which indicated the presence of replication-associated DSBs (Fig. 5C). Interestingly, when DNA2 was coexpressed with H-RAS, cells showed significantly reduced γ-H2AX foci formation in BrdUrd-positive cells, suggesting reduced levels of replication-associated DSBs. These cells consequently had increased BrdUrd incorporation, similar to that in the control cells (Fig. 5C).

On the basis of these observations, we tested whether coexpression of DNA2 would potentiate oncogenic effects of H-RAS activation. By using soft agar assay, we observed that coexpression of H-RAS and DNA2 significantly increased the number of colonies (Fig. 5D). Next, we found that H-RAS expression reduced cell proliferation and cells with DNA2 and H-RAS coexpression had increased cell proliferation (Fig. 5E). This result was further confirmed by using a second cell line expressing a different oncogene, *cyclin E* (Supplementary Fig. S3C). To summarize, our data revealed that an increase in

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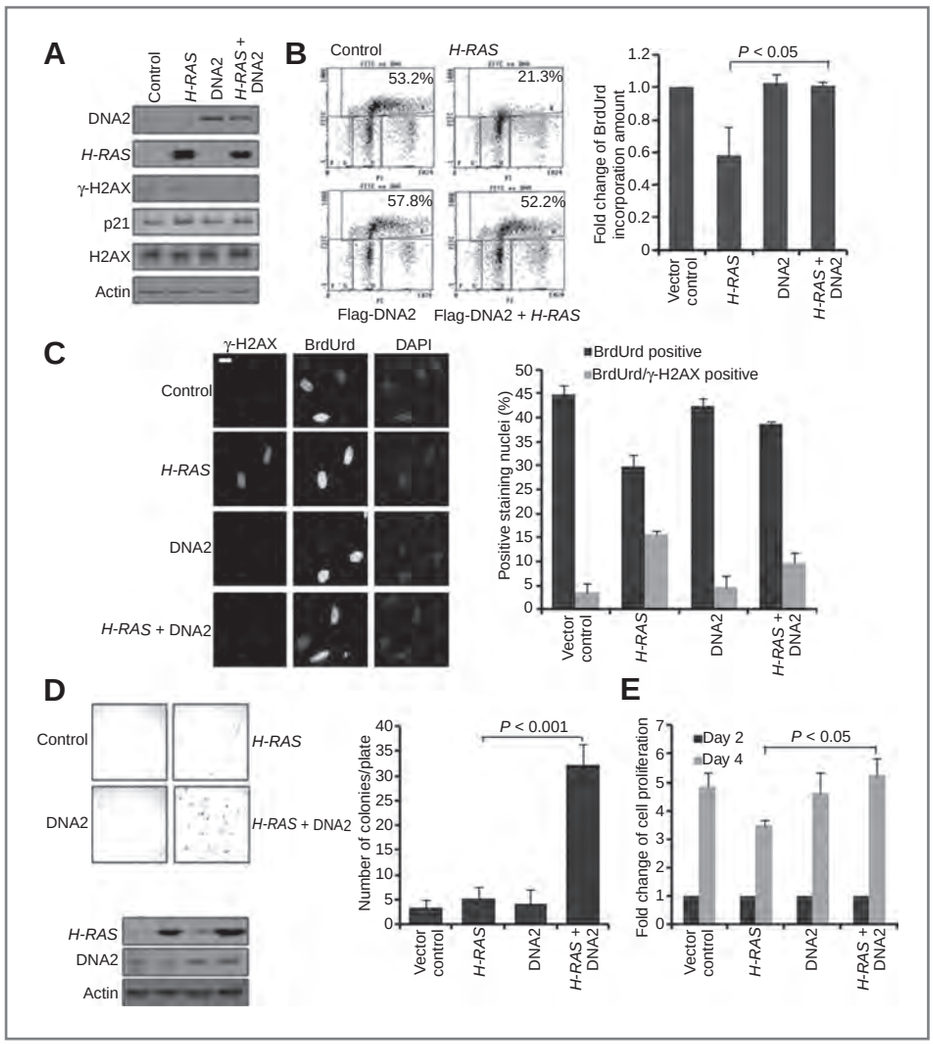

Figure 5. DNA2 alleviates replication-associated DSBs induced by oncogene activation U2OS cells were transfected with the indicated constructs. A, Western blot analyses. B, forty-eight hours later, cells were pulsed with BrdUrd (10 μmol/L) for 30 min. Left, flow cytometric profile. Right, quantitative summary. Each value is relative to the percentage of BrdUrd-positive cells in the control cells, which was set to 1 and represents the mean ± SD. C, seventy-two hours later, cells were labeled with BrdUrd (10 μmol/L) for 30 minutes before fixation. γ-H2AX and BrdUrd were co-stained in denatured conditions. Left, representative images (scale bar, 5 μm). Right, at least 50 cells were scored in each sample. Each value represents the mean ± SEM from 2 independent experiments. D, soft agar assay was conducted in MCF10A cells transfected with the indicated constructs. Left, representative images and Western blot analyses showing efficient transfection. Right, quantitative summary represents the mean ± SD. E, cell growth was analyzed by MTT assay. Each value is relative to the absorbance measured on day 2 (48 hours after transfection), which was set to 1 and represents the mean ± SD. DAPI, 4′,6-diamidino-2-phenylindole.

DNA2 expression level may promote HR repair and reduce accumulation of replication-associated DSBs induced by oncogene activation.

Clinical relevance of DNA2 in cancer

Given the function of DNA2 in alleviating replication stress induced by oncogene activation, we sought to address the clinical relevance of DNA2 in human cancers. First, by both Oncomine database search (39) and Western blot analysis, we found that cancer cells exhibited increased DNA2 mRNA expression (Fig. 6A) and protein expression (Fig. 6B). DNA2 expression was also upregulated in MCF10AT cells, which represent atypical hyperplasia, a premalignant disease observed early in the natural course of breast cancer development (Fig. 6C). We further examined DNA2 expression in a cell model representing a series of transitions from normal pancreatic ductal cells to cancer cells due to activating K-RAS (16). Again, we found that overexpression of DNA2 occurred at an early stage of transformation (Fig. 6C). More importantly, we found that DNA2 mRNA levels were significantly increased in a wide range of cancer types reported from independent research groups in the Oncomine database (ref. 39; Fig. 6D).

Pancreatic ductal adenocarcinoma has been found to almost always contain *K-RAS* mutations (>95% of tumors; 40). We specifically tested DNA2 expression in human tissues from pancreatic ductal adenocarcinoma. As we anticipated, DNA2 expression was increased in cancer tissues compared with adjacent normal tissues (Fig. 7A). We also found that DNA2 expression was positively correlated with the histologic grade of ovarian cancer (Fig. 7B). This finding suggested that elevated DNA2 expression might be functionally associated with increased intrinsic genomic instability during cancer development. To further test this possibility, we examined whether DNA2 expression exhibited a distinct pattern in different subtypes of breast cancer. On the basis of gene expression profiles, breast cancer can be divided into 5 subtypes: basal-like, Her2-positive, luminal A, luminal B, and normal breast-like (41). Morphologically, basal-like breast cancers have higher histologic grade than the other subtypes, and molecularly,

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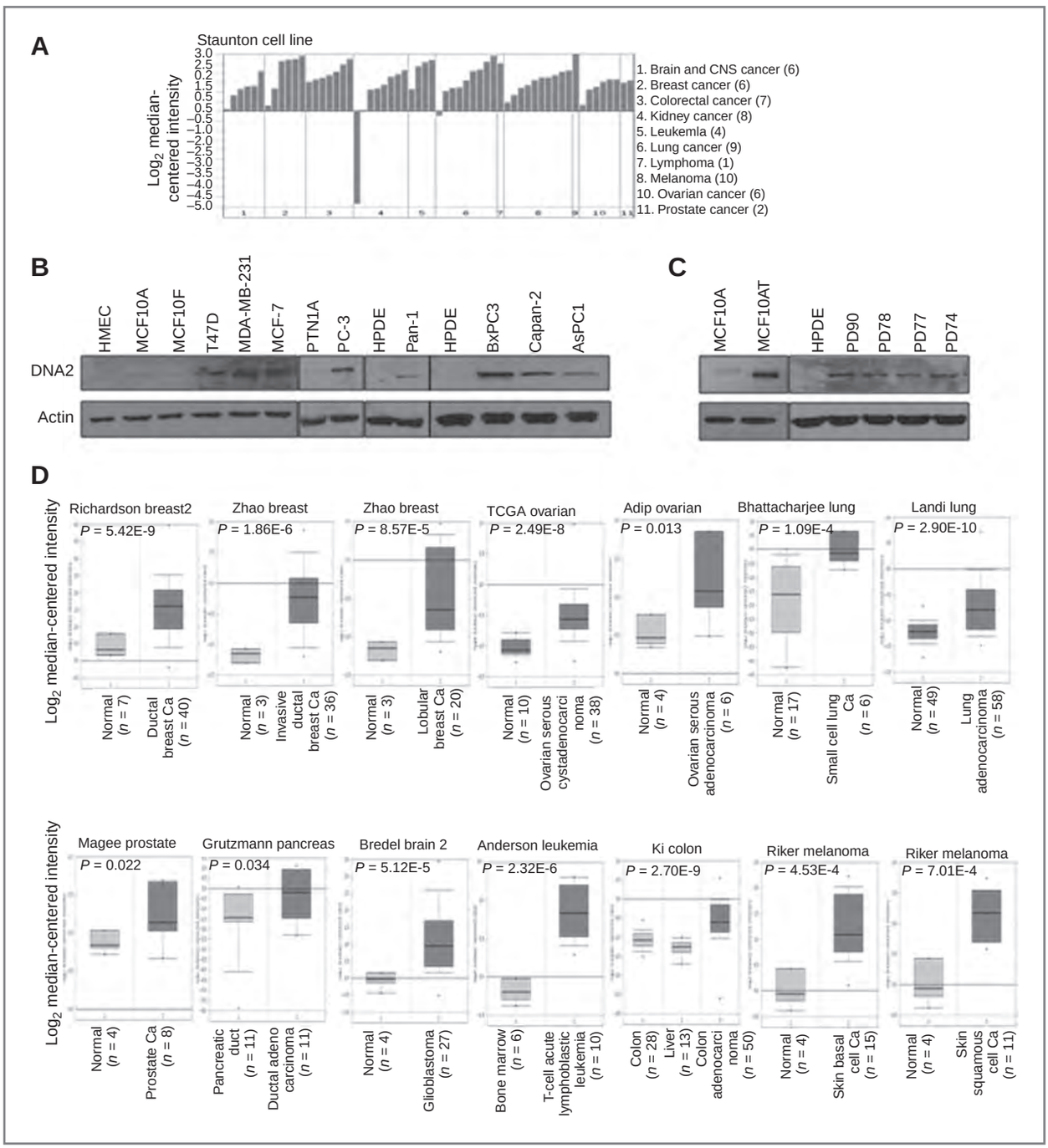

Figure 6. Enhanced DNA2 expression in human cancers. A, DNA2 mRNA levels in cancer cell lines. B and C, cell lysates from the indicated cancer cell lines were subjected to Western blot analyses. PD74, PD77, PD78, and PD90 cells represent a series of steps in oncogenic transformation of human pancreatic ductal epithelial (HPDE) cells. D, box plots showing DNA2 mRNA expression analyses. The mean expression values of DNA2 are shown for each group. Ca, carcinoma; CNS, central nervous system; HMEC, human mammary epithelial cells.

basal-like breast cancers exhibit a higher degree of genomic instability, which is manifested by increased numbers of mutations, translocations, and single-nucleotide polymorphisms (42). As we expected, DNA2 expression in breast cancer cohort (Netherlands Cancer Institute: NKI cohort, $n = 295$) was significantly higher in basal-like breast cancer than in the other subtypes (Fig. 7C). Indeed, in patients with breast cancer, DNA2 expression was positively correlated with the likelihood of breast cancer metastasis and was inversely correlated with the duration of overall patient survival (Fig. 7C).

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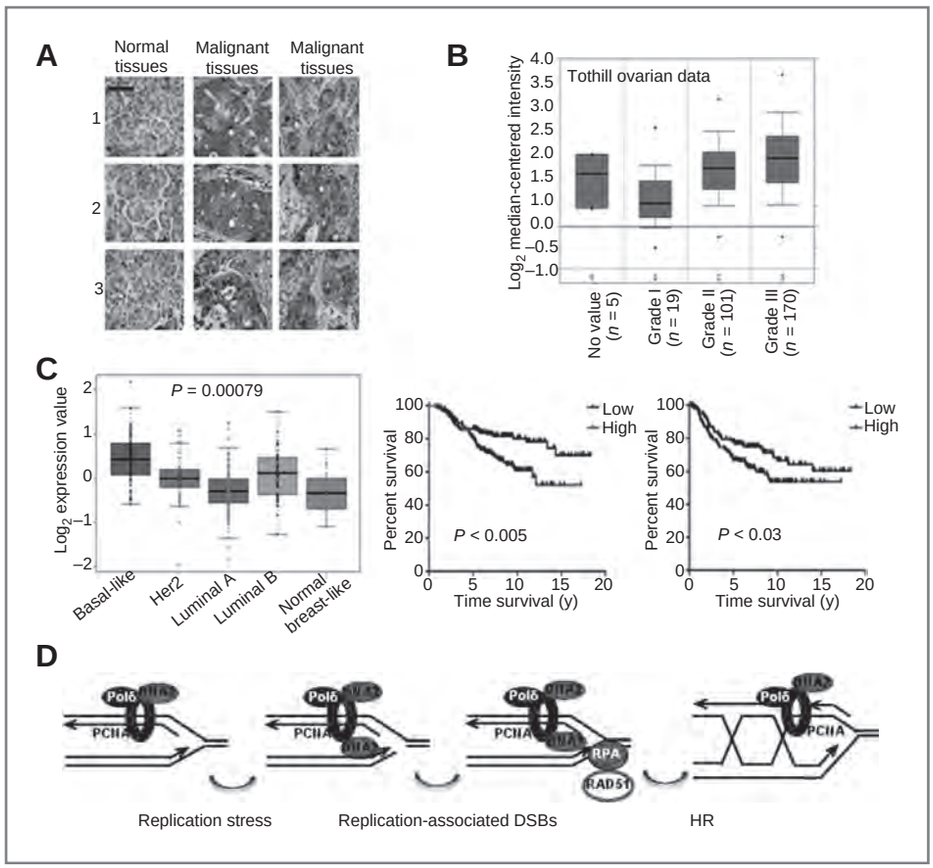

Figure 7. Clinical relevance of DNA2 in cancer. A, DNA2 protein expression in pancreatic cancer tissue samples (scale bar, 200 μm). B, DNA2 expression correlates with tumor grade in ovarian cancer. C, Left, box plot showing gene expression levels among breast cancer subtypes (295 breast cancers). Kaplan–Meier survival curves are shown for overall survival time (middle) and time to recurrence (right) stratified by expression levels of DNA2. D, proposed model for DNA2-mediated HR repair of replication-associated DSBs.

To further determine the biologic effects of enhanced expression of DNA2 in cancer cells, we depleted DNA2 in breast cancer MCF7 cells. DNA2 knockdown significantly increased DSB formation and reduced cell proliferation (Supplementary Fig. S4B). Interestingly, in another cancer cell line U2OS cells, which have competent DDR, DNA2-deficient cells were remarkably larger than control cells (Supplementary Fig. S4C) and exhibited a flat morphologic change. This observation led us to test whether DNA2 deficiency activates the senescence pathway, which permanently withdraws cells from the cell cycle. Compared with control cells, DNA2-depleted cells showed activation of γ-H2AX, phosphorylation and stabilization of p53, and activation of p21 (Supplementary Fig. S4D). DNA2 knockdown induced senescence as shown by β-galactosidase staining (Supplementary Fig. S4E). Notably, the number of senescent cells was remarkably reduced in the presence of aphidicolin (Supplementary Fig. S4E). Because DNA2 has been reported to regulate mitochondrial DNA replication and repair (22), we excluded the possibility that DNA2 depletion could cause mitochondria dysfunction and produce higher levels of reactive oxygen species (ROS), which could also contribute to cellular senescence (Supplementary Fig. S5).

Discussion

In summary, we propose a model in which DNA2 is associated with factors involved in DNA replication and accumulates at stalled or collapsed replication forks. One likely function of DNA2 nuclease is in ssDNA formation at stalled replication forks, which allows RPA and RAD51 loading and subsequent HR repair to restart the stalled replication forks. Thereby, DNA2 can alleviate replication stress, primarily by facilitating HR repair of replication-associated DSBs (Fig. 7D). Our finding that DNA2 interacts with polymerase δ, which is thought to be the main polymerase involved in lagging strand synthesis, is consistent with previous observations of DNA2 function in processing Okazaki fragment in yeast (43, 44). It is notable that compared with leading strand synthesis, lagging strand synthesis is associated with a greater risk of aberrant replication and genome instability because a large number of Okazaki fragments need to be processed and ligated (20). It is very likely that DNA2, besides resection, has a role in preventing excessive DNA damage by processing 5′ flaps formed during lagging strand synthesis or during HR. It is believed that DSBs are initially sensed by ATM, which is followed by end resection generating ssDNA for RPA loading and activation of ATR (35). As *Xenopus* DNA2 is found to be in a complex with ATM (21), whether the nuclease activity of DNA2 and the accumulation of DNA2 at DSB ends require ATM signaling requires further investigation.

Our data link the evolutionarily conserved function of DNA2 in HR repair to its role in alleviating both chemical- and oncogene-induced replication stress (2–5). During

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tumorigenesis, excessive growth signals often lead to hyperproliferation of cancer cells, which results in replication stress and increased formation of replication-associated DSBs. In our study, we have identified that enhanced DSB resection activity such as mediated by increased DNA2 expression may facilitate HR repair, which constitutes a key step to enhance cellular tolerance of replication-associated DSBs and provides cancer cells with a survival advantage. Currently, by using a mouse genetic interaction approach, we are establishing a DNA2 conditional knockout mouse model to test this hypothesis *in vivo*.

We speculate that our findings may have significant clinical implications for cancer management. First, DSBs associated with oncogene-induced replication stress are observed in large fractions of early lesions from various human cancer tissues. Activation of the DSB end resection pathway such as DNA2 overexpression might serve as a marker for genetic alterations in premalignant lesions that promote tumor progression. Second, our findings raise the intriguing possibility that nucleases/helicases involved in DSB end resection, which are highly expressed in a wide range of human cancers, could serve as a new class of therapeutic targets. Indeed DNA nucleases/helicases are intensively studied as potential anti-cancer targets. Lately, a small-molecule inhibitor of the WRN helicase was identified, therefore similar strategy should be possible for DNA2 enzyme (45–48). Third, recent studies showed excellent response of *BRCA1*/*BRCA2*-mutated tumors with HR repair deficiency to PARP inhibitors (49, 50). Currently, multiple clinical trials are testing the efficacy of PARP inhibitors in treating triple-negative breast cancer, which clinically and molecularly resembles *BRCA1*/*BRCA2*-mutated tumors (51, 52). Our data showed that basal-like breast cancer, which largely overlaps with triple-negative breast cancer, had significantly higher expression levels of DSB end resection factor DNA2. These factors and the activity of DSB end resection might provide a candidate biomarker to predict the response of triple-negative breast cancer to PARP inhibitors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: G. Peng, R. Y.-L. Tsai, G. Ira, S.-Y. Lin
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Peng, Y.-Y. Park, J.-S. Lee
Writing, review, and/or revision of the manuscript: G. Peng, G. Ira, S.-Y. Lin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Peng, W. Zhang, M.-R. Pan
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